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TOXICITY OF DECON GREEN TO *Ceriodaphnia dubia*, *Daphnia magna*, AND *Vibrio fischeri*

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14. ABSTRACT

The U.S. Army is developing a hydrogen peroxide-based decontaminating solution [Decon Green (DG)] that is effective against chemical, as well as biological agents. The components of the decon solution are less hazardous to the user than standard decon solutions (i.e., DS-2/DAM). Toxicity data exist on the individual components; however environmental information on the mixture is lacking. This report addresses the aquatic toxicity of DG, presenting studies that will provide baseline aquatic toxicity screening levels on neat DG solution. Although a number of aquatic organisms are available for short-term testing, the following organisms were used to estimate the aquatic toxicity: *Daphnia magna* (freshwater crustacean, water flea), *Ceriodaphnia dubia* (fresh water crustacean), and *Vibrio fischeri* (marine luminescent bacteria). The 5-min EC50 for V. *fischeri* was 2.0 x 10^{-2}% vol/vol, and the 48-hr for *D. magna* and *C. dubia* were EC50s 2.6 x 10^{-3} and 2.5 x 10^{-3}% vol/vol, respectively. The no observable effects concentration for *C. dubia* reproduction was 1.6 x 10^{-3}% vol/vol. Based on the aquatic toxicity, DG is a better candidate decontaminating solution than DS-2 or DAM; however, unlimited release into the aquatic environment is not advised.

15. SUBJECT TERMS

Decon Green	Aquatic Toxicity	*Daphnia magna*
EC50	Microtox	*Ceriodaphnia dubia*

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PREFACE

The work described in this report was authorized under Project No. 2E2RDF, Decon Green Project. This work was started in June 2002 and completed in September 2003.

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1. INTRODUCTION

The U. S. Army is developing a hydrogen peroxide-based decontaminating solution, Decon Green (DG) that is effective against chemical, as well as biological agents. The components of DG are much less hazardous to the user than current decon solutions such as DS-2. A component of the DS-2 solution (ethylene glycol monmethyl ether, EGME) has been determined to cause birth defects, fetotoxicity and bone marrow complications in laboratory animals.[1] Also, DS-2 is highly corrosive material creating compatibility problems.

Decon Green is a proposed candidate decontaminating solution for which there is limited environmental information. Safety data sheets and open literature publication exist on the individual components. However, environmental information on the mixture is lacking. Using the information provided on individual components can only provide speculation on environmental effects and does not consider the possible synergistic or antagonistic effects.

The studies described in this report will provide baseline toxicity screening levels on neat Decon Green solution. The information presented in this report can be used to assist in the preparation of Environmental Assessments (EA), which are needed before a new candidate can be fielded. This study does not address the possible change in toxicity due to the method of deployment or property changes resulting from agent neutralization.

Although a number of aquatic organisms are available for short term testing, the *Daphnia magna* (freshwater crustacean, water flea), *Ceriodaphnia dubia* (fresh water crustacean), and *Vibrio fischeri* (marine luminescent bacteria) were chosen as the primary test organisms. Bioassays with these target organisms were selected on the basis of their ability to determine chemical toxicity to ecologically relevant species and because they include at least one reproduction or growth component among the measurement endpoints. Because these species are used nationwide, an extensive data base exists for toxicity comparisons. Also, these test organisms are inexpensive to culture in the laboratory, and cultures can be maintained indefinitely when proper care is exercised.

The DG formulation used during testing in this report is listed in Table 1. The mixing procedures for preparing the DG solution is described in detail in the methods section.

Table 1. Decon Green Formulation

Potassium molybdate	0.02M
Potassium carbonate	0.15M
Hydrogen peroxide (35 %)	30 vol%
Propylene carbonate	60 vol%

2. METHODS AND MATERIALS

2.1 Decon Green Mixing Procedure.

Decon Green was freshly prepared for each study in 100 mL batches. The solid components were placed into a 250 mL beaker (0.47 g potassium molybdate and 2.1 g potassium carbonate). Propylene carbonate (60 mL) was added to the solids and swirled while being placed into a sonic water bath for 15-20 sec. This was done to assure the solid materials did not clump and form dry pockets. Triton X-100® (10 mL) was then added and swirled while in the sonic water bath for an additional 15-20 sec. The final mixing step included the addition of 30 mL of 35 % hydrogen peroxide. The solution was swirled to mix the remainder of solid particulate (H_2O_2 was always added last). When the H_2O_2 was added, the solution turned from cloudy white to a redish brown (Figure 1). The solution was allowed to sit for 30 min before being used. The pH of the Decon Green solution was approximately 7.1. All concentrations referred to in this report are nominal and were not confirmed with analytical determinations.

The water used to dilute DG and grow *D. magna* and *C. dubia* was obtained from a 400 ft deep well. The water was past through a micronizer (air injection system), limestone pH adjustment tank, iron removal system, charcoal filtration, particulate filtration, and UV sterilization. For quality control monitoring, water samples are sent to an independent laboratory for analysis of 96 groundwater pollutants twice yearly.

2.2 Microtox Test Procedure.

The Microtox (MTX) bioassay exposes a bioluminescent marine bacterium (*Vibrio fischeri*) to a sample of unknown toxicity and measures the change in light output as the means of determining effects on the organisms. The reduction in light output is a direct indication of metabolic inhibition. The bacterium was cultured by Azur Environmental (Carlsbad, CA)* and shipped in lyophilized form. The bacterium (stored frozen) was re-hydrated immediately before testing. Each bioassay used < 3 mL of sample and was performed in a temperature controlled photometer. Decon Green samples were diluted to 0.3 % using MTX diluent. Salinity and pH adjustments were not needed after dilution. The assays were performed in glass cuvettes containing 1 mL of sample. For optimum accuracy in predicting toxicity, the assay must have a minimum of four dilutions exhibiting a dose response. At 5 and 15 min, the control and treatment groups were measured for light output. Data were analyzed using the MTX test protocol software to determine the EC_{50} (the effective concentration causing a 50% reduction in light output).

2.3 *Daphnia magna* Bioassays.

Daphnia are freshwater crustaceans that constantly filterfeed at a rate of 2.8 mL/hr^2 on particulate matter suspended in the water column. These organisms swim throughout the water column with the aid of a secondary antenna. Daphnia are easily maintained

* AZUR Environmental, 2232 Rutherford Road, Carlsbad, CA.

in laboratory cultures and are employed in toxicity screening worldwide. Testing requires small sample volumes and space utilization is minimal.

Daphnia were originally obtained from Dr. Freida Taub, University of Washington (Seattle, WA), and cultured using techniques described by Goulden et al.[3] Culture/dilution media was supplied from well water that was passed through a treatment system containing a micronizer (air injection), limestone pH adjustment, iron removal system, carbon filtration, and UV sterilization. Daphnia were fed a mixture of vitamin enriched algae, *Selenastrium capricornutum*, *Ankistrodesmus falcatus*, and *Chlamydomonas reinhardti*, obtained from R. O'Neil,* University of Texas at Austin (Austin, TX) Culture Collection. Daphnia reared from third generation post acclimated adults were used in testing. Neonates (Daphnia < 24-hr old) were placed in 250 mL glass beakers, containing 100 mL of sample. Decon Green (100 % stock) was diluted using well water as described above. Beakers were placed into a temperature controlled room at 20 °C, with a light:dark cycle of 16:8. All testing conformed to Environmental Protection Agency (EPA) standard guidelines.[4] At 24 and 48 hr, the daphnia were checked for immobilization by gently touching the daphnids with a pasture pipette. If the daphnia could not swim actively for 15 sec they were considered immobilized.

2.4 Ceriodaphnia Chronic Bioassays.

The chronic bioassays were based on EPA standard guidelines.[5] The *Ceriodaphnia dubia* were obtained from the University of Maryland, Wye Research and Education Center (Queenstown, MD). The ceriodaphnia were grown in well water passed through the treatment system described above. However, the well water was then diluted with distilled water to produce a final hardness of 90 ppm. The Ceriodaphnia were maintained as batch cultures in 800 mL of media. The batch cultures were maintained for 14 days while initiating new cultures every 5-7 days. Ceriodaphnia were fed a mixture of *Selenastrum capricornutum* (green algae) and cerophyl extracts. The algae were grown in vitamin enriched media[6] for approximately 7 days before being harvested and fed to the ceriodaphnia at a concentration of 10^6 cell/mL. The cerophyl (dehydrated cereal of grass leaves) stock solutions were prepared by suspending 3.75 g of cerophyl in 500 mL of distilled water. The mixture was placed into a blender at high speed for 5 min. The solution was allowed to stand over night in a refrigerator to settle out the large particulate. The supernatant was then decanted and stored frozen in 40 mL aliquots. Cerophyl was added to the media at a concentration of 1 mL/100 mL of media. Each batch of media was then aerated for 24 hr before being used.

Approximately 2 weeks before testing, 25 adults were isolated from the batch cultures for offspring production. The second brood produced was grown to adult stage for production of offspring (<24 hr old). These offspring were used in toxicity testing.

All glassware used for testing and culturing was washed with non-phosphate soap, rinsed with tap water until all soap residue was removed, rinsed twice with distilled water, and heated to approximately 465 °C for 2 hr.

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* O'Neil, R., University of Texas at Austin, Austin, TX.

One batch of Decon Green (100% stock) was prepared and used for media renewal for the entire test. Samples from the 100% Decon Green stock were volumetrically diluted to 0.1% using ceriodaphnia media. The 0.1% stock was diluted to the desired test concentrations (3.2, 2.4, 1.6, 0.8, 0.4 x 10^{-3} %). The test chambers consisted of 30-mL glass beakers containing 15 mL of dilute sample. There were 10 replicates for each treatment and control containing one individual each. The media was changed and fresh food added daily. Mortality, reproduction, pH, hardness, conductivity, and dissolved oxygen were recorded daily. The light cycle was maintained a 16-hr light/8-hr dark. The light intensity was maintained at approximately 90 ft-c,* with a room temperature of 25 °C. Acceptable test criteria were met when 80% of the control organisms survived and 60% of the control organisms had three broods totaling 15 or more offspring.

2.5 Statistical Evaluation.

Point estimation of EC_{50} (the effective concentration that immobilizes 50% of the organisms) calculations were performed using the Probit Analysis contained in the Minitab™ (Minitab, State College, PA)† statistical software package. The IC_p (the concentration that causes a reduction in offspring production) was calculated using a linear interpolation method for calculating inhibition concentrations.

Survival and reproduction data were subjected to hypothesis testing, to determine the No Observable Effects Concentration (NOEC) and the Lowest Observable Effects Concentration (LOEC). Survival data were subjected to Fisher's Exact test to determine if there were any significant survival differences at the 95% confidence level between control and treatment groups. Reproduction data were subjected to One Way Analysis of Variance (ANOVA) to determine significant differences at the 95% confidence level between control and treatment groups.

Treatment groups having no survival in any replicates were excluded from the NOEC and LOEC reproduction calculations. However, the treatment groups having no survival were included in the calculation of the EC_{50} and IC_p endpoints.

3. RESULTS

Decon Green (DG) was prepared and allowed to stand for 30 min before being used in testing. This allowed time for any remaining particulates to dissolve. The solution was quite reactive as shown in Figure 1. The bubbles from the hydrogen peroxide off-gassing was seen forming at the surface of the solution. Off-gassing made sample transfer difficult. Pipettes were rinsed several times with DG to reduce off-gassing to maintain desired volume for transfer. At 16 to 17 days, the DG solution separated into two distinct layers. The top layer was clear and the bottom layer was pale clear yellowish in color.

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* ft-c – foot-candles

† Minitab Inc., 3081 Enterprise Drive, State College, PA.

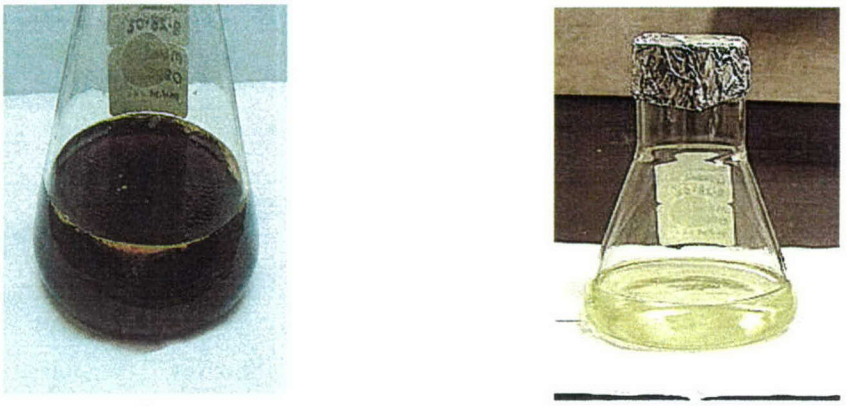

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Figure 1. Decon Green After Additions of H_2O_2. A. Decon Green 30 min after the addition of H_2O_2. B. Decon Green 7 days after the addition of H_2O_2.

3.1 Microtox Results.

Overall, *Vibrio fischeri* (microtox assay) was less sensitive to DG than *D. magna* and *C. dubia* by approximately one order of magnitude. Table 2 lists EC_{50} values for DG at 0, 6, 16, and 34 days after mixing. At day 16, the toxicity had decreased approximately an order of magnitude, and thereafter, remained unchanged up to 34 days.

Table 2. Microtox Results Using Decon Green With Comparisons to DAM and DS-2

Sample	Age of Sample (days)	5 min EC50 (% vol/vol)	15 min EC50 (% vol/vol)
Decon Green	0	2.0×10^{-2} %	2.0×10^{-2} %
Decon Green	6	6.0×10^{-2} %	7.0×10^{-2} %
Decon Green	16	1.0×10^{-1} %	1.0×10^{-1} %
Decon Green	34	1.0×10^{-1} %	1.0×10^{-1} %
DAM[8]	0	5.3×10^{-4} %	
DAM	7	5.6×10^{-3} %	
DAM	14	8.0×10^{-3} %	
DS-2[9]	0	4.0×10^{-3} %	
DS-2	7	4.0×10^{-3} %	
DS-2	14	4.6×10^{-3} %	
Malathion[10]		2.4×10^{-6} %	
Phenol*		1.8×10^{-3} %	
Acetone*		2.3 %	
Methanol*		5.6 %	

* Work conducted at ECBC

** Minitab Inc., 3081 Enterprise Drive, State College, PA.

Other decon solutions [Decontaminating Agent Multipurpose (DAM) and Decontaminating Solution 2 (DS-2)] were included in Table 2 for toxicity comparison. At time 0, DG was approximately two orders of magnitude less toxic than DAM and an order of magnitude less toxic than DS-2. At approximately 1 week, DG was an order of toxicity less toxic than DAM and DS-2. Malathion, phenol, acetone, and methanol were included in Table 1 as reference toxicants.

3.2 *Daphnia magna* Results.

Daphnia magna was one order of magnitude more sensitive to DG than *Vibrio fischeri*. The 24- and 48-hr EC_{50} were 2.8 and 2.6 x 10^{-3} %, respectively. Table 3 lists EC_{50} values for *D. magna* exposure to DG along with toxicity values for DS-2 and DAM decon solutions. Decon Green was two orders of magnitude less toxic to *D. magna* than DAM and approximately 1.5 times less toxic than DS-2.

Table 3. EC_{50} Values for D. Magna Exposure to Decon Green with Comparisons to Other Decon Solutions

Sample	24 hr EC_{50} (% vol/vol)	48 hr EC_{50} (% vol/vol)
Decon Green	2.8 x 10^{-3} %	2.6 x 10^{-3} %
DAM[6]	---	5.0 x 10^{-5} %
DS-2*	---	1.7 x 10^{-3} %
Malathion[8]	---	8.0 x 10^{-8} %

*Minitab Inc., 3081 Enterprise Drive, State College, PA.

3.3 Ceriodaphnia Results.

A 100 mL batch of DG was prepared and used throughout the 7 days of ceriodaphnia testing. This was done to mimic a one time spill and include any toxicity influence of possible degradation products during the testing period. The control ceriodaphnia met the testing criteria by having > 80% survival and > 60% having three broods of offspring totaling over 15 individuals. In Figure 2, the average number of offspring per adult per treatment group is shown. At 0.4 x 10^{-3} % (vol/vol), there was a slight increase in offspring productivity, however, this was not biologically significant ($p \leq 0.05$). The NOEC for ceriodaphnia reproduction was 1.6 x 10^{-3} % (vol/vol). The LOEC for reproduction was 2.4 x 10^{-3} % (vol/vol).

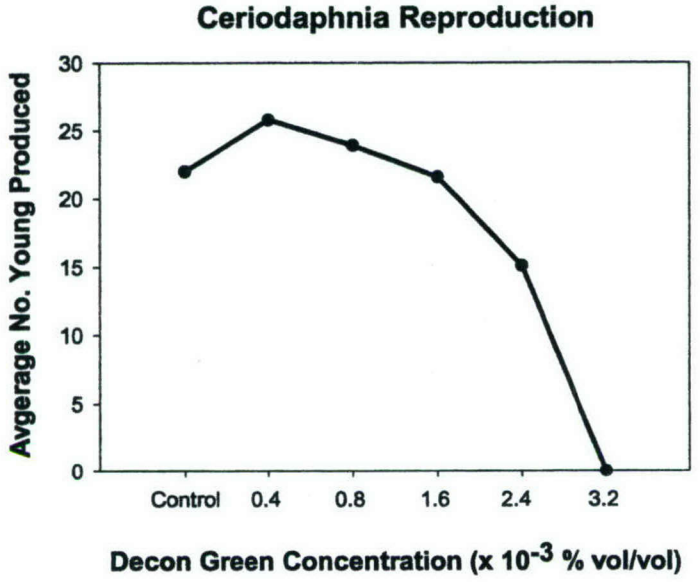

Figure 2. Average Number of Offspring Produced at 7 Days Per Treatment Group

There were no significant differences in ceriodaphnia acute toxicity at 24 hr, 48 hr, and 7 days. All mortality occurred within the first 24 hr (EC_{50} = 2.5 x 10^{-3} %). The NOEC for survival was 2.4 x 10^{-3} % (vol/vol), and the LOEC was 3.2 x 10^{-3} % (vol/vol). The IC_{20} (the concentration that inhibited reproduction to 20% of the control was 1.8 x 10^{-3} % vol/vol (95% C.I. = 1.2 –1.9 x 10^{-3} %).

4. DISCUSSION

The toxicity results were ranked using the Chemical Scoring System for Hazard and Exposure Identification.[9] This system was typically used in the preliminary screening process and was not intended to be a substitute for risk assessment. The system assigns a score based on the acute (≤ 96-hr) toxicity data and/or chronic NOEC toxicity data. The toxicity units used in this system were presented in parts per million (ppm). Using the density of DG (1.17 g mL^{-1}, in-house determination), the data was transformed to ppm and scored (Table 4). The scoring system developed by O'Bryan and Ross does not rank the scores using common terms typically used in mammalian toxicity rankings. The U.S. Fish and Wildlife Service (USFWS) published a Research Information Bulletin[10] suggesting relative aquatic toxicity terms based on EC_{50} data. The ranking system considers EC_{50} results > 1000 ppm to be "Relatively Harmless" and results < 0.01 ppm as "Super Toxic." Similar descriptive rankings are used by Kamrin.[11] In Table 4, the toxicity was scored and ranked based on the EC_{50} results from these aquatic bioassays. There were no guidelines given for ranking the NOEC results using rankings provided by Kamrin.

Table 4. Toxicity Scoring of Decon Green Using O'Bryan and Ross, Chemical Scoring System for Hazard and Exposure Identification and Ranking Using USFWS System

	EC_{50} (ppm)	Score (1-9, 9 being most toxic)	Ranking (16)
Vibrio fischei (5 min)	350	2	Practically Nontoxic
D. magna (48 hr)	30.4	5	Slightly ToxicSlightly
C. Dubia (96 hr)	29.3	5	Toxic
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C. Dubia (NOEC)	28.1	5	

The scoring protocols dictate that when multiple scores are assigned in the acute and chronic category, the highest score should be selected as the aquatic toxicity score. Using the Chemical Scoring System for Hazard and Exposure Identification, DG directly amended into water had an aquatic toxicity score of 5 (slightly toxic). In comparison, Table 5 lists the score and ranking for acetone using data from *Vibrio fischei* (microtox), *D. magna*, and *C. dubia*. The overall score for acetone directly amended into water was 1 which was ranked as "Relatively Harmless." Decon Green had a score four units higher in toxicity than acetone. At the other end of the scoring scale, the 48-hr EC_{50} for malathion was approximately 0.002 ppm (19, 20) for *D. magna* and *C. dubia*. Malathion scores a 9, which ranked "Super Toxic." Decon Green was more toxic than acetone yet orders of magnitude less toxic than malathion.

The ceriodaphnia tests were conducted using a single batch of DG throughout a 7-day period. This was done to incorporate any toxic influence that may be produced from degradation by-products, simulating a one time spill directly into water. During ceriodaphnia testing, all mortality occurred within the first 24 hr. If DG was allowed to degrade before being used in testing, the overall toxicity to ceriodaphnia may be reduced. Similar studies were conducted using the Microtox assay (Table 2). Decon Green was prepared and allowed to stand for up to 34 days. Assays were run on the same batch at 0, 6, 16, and 32 days. After 6 days, the toxicity was reduced by half. After 16 days, the toxicity of DG to *Vibrio fischei* (microtox) was reduced to "Relatively Harmless" levels.

Using the Chemical Scoring System for Hazard and Exposure Identification, DG scored a 5 indicating that it is considered to be "slightly toxic." However, during field application over-spray may quickly reach concentrations that will cause very toxic conditions. The reader should not consider a ranking of "slightly toxic" a green light to release unlimited quantities of DG into the environment. There is an unusually narrow margin between the NOEC and the 50% mortality concentration. Due to this narrow range, there is a minute safety factor between the NOEC and the 50% mortality concentration. Procedures should be employed to contain as much Decon Green from release into the environment as economically possible.

Table 5. Toxicity Scoring/Ranking for Acetone

	EC_{50} (ppm)	Score (1-9, 9 being most toxic	Ranking (16)
Vibrio fischei (5 min)	18,170	1	Relatively Harmless
D. magna (48 hr)	9,218[15]	1	Relatively Harmless
C. Dubia (96 hr)	8,098[15]	1	Relatively Harmless

The research presented in this paper represents DG added directly into water. Decon Green is intended to be used for the decontamination of equipments and most likely will not be sprayed directly into a water body. The toxicity results presented in this paper does not incorporate the effects of soil/DG interaction, nor in any way assess the terrestrial toxicity of DG.

5. CONCLUSIONS

Based on acute aquatic toxicity, neat Decon Green (DG) is less toxic to *Daphnia magna* and *Vibrio fischeri* than DS-2 and DAM decon solutions. Using the Chemical Scoring System for Hazard and Exposure Identification, the overall aquatic toxicity score for DG was 5, which was slightly toxic to aquatic organisms. The safety factor between the No Observable Effects Concentration and the EC_{50} (mortality concentration) is extremely narrow. Testing is needed to provide insight to how soil/vegetation interaction may affect the aquatic toxicity of DG. Information on toxicity of DG to soil dwelling organisms and terrestrial vegetation is not available, and terrestrial bioassays should be included in future testing.

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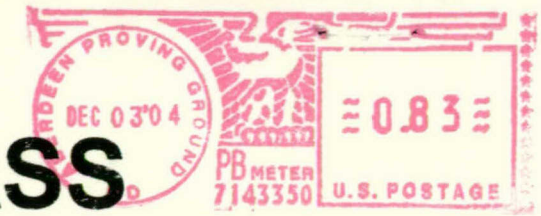

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